Supplementary Figure 1

Light path in the inverted light-sheet microscope.

Cross section through a model of the microscope’s sample mounting system: dash-dot lines indicate axis of excitation (Ex) and emission (Em) light paths, mirrors (M), illumination objective (IL), imaging objective (IM), flexible sealing ring (SR), immersion water reservoir (W), mounting base plate (BP).
Supplementary Figure 2

Environmental control incubator on the inverted light-sheet microscope.

Model of microscope’s sample mounting system with an incubator enabling precise temperature control as well as control of CO$_2$ concentration in the atmosphere. Note the bottom plate of the incubator (shown in black) that minimizes heat transfer to the optical table. Openings (not shown but indicated by dashed line) on top and on the side of the incubator are fitted with sealed doors (not shown on the model).
Supplementary Figure 3

Light path of the inverted light-sheet microscope.

Schematic representation of inverted light-sheet microscope light path: lasers (L1, L2), acousto-optical modulator (AOM), clean-up filter (CF), glass plates (GP), kinematic mirrors (KM), dichroic mirrors (DM), optical fiber (F), filter wheel with neutral density filters (FW), polarizing beam splitters (PBS), fixed mirrors (M), beam expander (BE), mechanical shutters (SH), galvanometric scanning mirror (SM), scan lens (SL), tube lenses (TL), excitation light (Ex), illumination objective (IL), imaging objective (IM), motorized filter wheel with emission filters (MFW), emitted light (Em), field stop (FS), relay lenses (RL1, RL2), emission filters (EM1, EM2), sCMOS camera (sCMOS). Position of the EGFP and mCherry channels on the camera chip is indicated in inset.
Supplementary Figure 4

Point spread function of the inverted light-sheet microscope.

(a) Point spread function of the inverted light-sheet microscope system obtained by averaging images of fluorescent beads. (b) Intensity profile of the point spread function along the x- and y-axis.

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Supplementary Figure 5

Live mice born after transfer of imaged embryos.

(a) Live-cell time-lapse imaging of mouse pre-implantation embryos expressing H2B-mCherry (red) and mG (green). Maximum intensity projections of first and last time-frames along the z-axis are shown. The cell surface marker was only imaged for the final division from 32 to ~64 cells. (b) Mice born after imaged embryos were implanted into pseudopregnant CD-1 females. Note that variable coat color is expected because imaged embryos are F2 hybrids from C3H and C57BL/6 strains.
Supplementary Figure 6

Confocal time-lapse imaging of pre-implantation embryos.

(a) Live-cell time-lapse confocal microscope imaging of mouse pre-implantation embryos expressing H2B-mCherry (red) and mG (not imaged). Maximum intensity projections of the first and the last time-frames along the z-axis and transmitted light images are shown. (b) Transmitted light pictures of imaged embryos and non-imaged control embryos kept under identical conditions. Note that because imaged embryos were arrested the time-lapse was stopped and mG transgene was not imaged.
Supplementary Figure 7

Lineage trees and fate assignment.

Lineage trees of mouse pre-implantation development from the one or two cell stage to the blastocyst for all tracked embryos (n = 12). Percentage of TE (red) or ICM (blue) cells derived from each cell is displayed on the tree by color code. All occurrences of cell death have been manually annotated and are indicated by terminated branches.
**Supplementary Figure 8**

Statistical analysis of lineage segregation.

Simplified representation of lineage trees. Color code represents how much a relative contribution of each branch to the ICM or TE lineages deviates from the whole embryo average (white, visible at one cell stage). Branches contributing only to ICM are shown in red and branches contributing only to TE are shown in blue. Probability that observed lineage segregation at 2-, 4-, 8-, 16- or 32-cell stage occurred by chance is shown on the right side of each tree. Probability was estimated using a permutation test where the observed tree was compared to 100,000 trees with randomized fates (Online Methods and Supplementary Note).
Supplementary Figure 9

Orientation of division axis correlates with cell fate.

(a) Live-cell time-lapse imaging of embryos expressing H2B-mCherry (white) and mG (not shown). Maximum intensity projections of selected time-points of the same embryo are shown. Examples of cells that contribute only to ICM (red), only TE (blue) or both (grey) lineages are shown together with their trajectories. Angle between the division axis and a line parallel to the embryo surface is illustrated by colored circle arcs. Cells dividing inwards have a positive and cells dividing outwards a negative directional division angle (Online Methods). Estimated embryo outline and center are shown by dashed circle and a cross. (b-d) Distribution of angles measured as illustrated in a. Cells were split at 16-cell stage into three categories depending on their contribution to cell lineages and analyzed separately. Cells that give rise only to ICM are shown in b, both lineages in c and only to TE in d. Random distribution is indicated by black line in each graph. Color code in graphs represents cells contributing to ICM (red), TE (blue) or both (grey) lineages at either 16 or 32-cell stage.
Supplementary Figure 10

Orientation of divisions at the transition from 8- to 16-cell stage is not random.

Distribution of division angles (Online Methods) during cell divisions from 2-cell stage up to 32-cell stage. Mitoses where cells divided parallel to the embryo surface have an angle of division 0°, whereas mitoses where cells divided in the direction of the inside/outside axis have a division angle of 90°. Random distribution is indicated by a black line. Probabilities that observed angles are coming from this random distribution are shown inside the graphs (Kolmogorov–Smirnov test, sample size inside graphs).
Cells that contribute exclusively to TE or ICM lineage are preferentially coming from divisions along inside/outside axis.

Distribution of division angles (Online Methods) during cell divisions from 8- to 16- and 16- to 32-cells stage. Mitoses where cells divided parallel to the embryo surface have an angle of division 0°, whereas mitoses where cells divided in the direction of inside/outside axis have an angle of division 90°. Cells were split at 16-cell stage into three categories depending on their contribution to cell lineages and analyzed separately. Cells that give rise only to ICM are shown in the top two graphs, both lineages in the middle graphs or only to TE in the bottom two graphs. Random distribution is indicated by black line in each graph. Probabilities that observed angles are coming from this random distribution are shown inside the graphs (Kolmogorov–Smirnov test, sample size inside graphs).
Supplementary Figure 12

Cell fate can be predicted from directionality of cell divisions.

Schematic representation of simple rules that enable correct prediction of the fate of 84% of cells based solely on directional division angle (Online Methods). Progeny of all cells that divide at the 8- to 16-cell stage transition at an angle larger than 40° to the embryo surface will become ICM (top bar red, inside daughters) or TE (top bar blue, outside daughters). Fate of remaining cells (top bar grey) will be specified in next cell cycle where only inside daughters from divisions with an angle larger than 40° to the embryo surface will become ICM (bottom bar red). Remaining cells will become TE (bottom bar blue). Note that cells at 32-cell stage where one of the daughter cells dies or that gave rise to one TE and one ICM cell at 64-cell stage were not included in the analysis (7.8% of all cells).
Supplementary Results

Analysis of division angle orientation and cell fate

To further mine the 4D imaging data we projected the percentage of TE and ICM progeny at each time point onto each blastomere in the 3D movies of the developing embryos by color-coding their nuclei (Fig. 3b), providing real-time visualization of cell trajectories and fate contributions in live mammalian embryos (Supplementary Video 4). One of the mechanisms that have been suggested to contribute to fate specification is the orientation of the blastomere division axis relative to the embryo surface\(^1\)\(^-\)\(^3\). Visual inspection indeed suggested that an inward directed anaphase trajectory at the 8- to 16- or 16- to 32-cell division correlates with future ICM fate of the progeny of the respective daughter cell, while an outward directed trajectory seemed to correlate with TE fate (Supplementary Video 4). We therefore systematically mined all 4D trajectory data for division angles at anaphase. This analysis revealed that the spindle axes in the embryos became significantly non-random with a bias towards perpendicular orientation to the embryo surface for the critical 8- to 16-cell division (Supplementary Fig. 10), consistent with earlier observations\(^2\). 16-cell stage blastomeres whose progeny would become exclusively ICM were almost all derived from the inside directed daughter cell from a division with a close to perpendicular axis, while blastomeres whose progeny would only become TE came mostly from the outside-directed daughter cell (Supplementary Figs. 9a,b,d and 11, and Supplementary Video 5). Once inside the embryo, ICM progenitor cells no longer showed a bias in division orientation, while cells on the outside contributing exclusively to TE divided preferentially parallel to the embryo surface in the 16- to 32-cell stage division (Supplementary Figs. 9a,b,d and 11, and Supplementary Video 5). In contrast, blastomeres that at 16-cell stage still contributed to both TE and ICM lineage were derived from division without any bias in division orientation. These cells, however, underwent 16- to 32-cell stage divisions with slight but significant bias towards perpendicular orientation to the embryo surface (Supplementary Figs. 9c and 11, and Supplementary Video 5).

Using a single threshold of 40\(^\circ\) (90\(^\circ\) being perfectly inward and -90\(^\circ\) perfectly outward directed; Supplementary Fig. 9a) applied on the division orientation at 8- to 16- or 16- to 32-cell transitions we could correctly predict a fate of 84% of the progeny of these two divisions (Supplementary Fig. 12).

Overall, our data supports a two-step sorting model of first cell fate specification in the early mouse embryo. At the decisive 8- to 16-cell stage transition, after which cells for the first time show bias towards TE or ICM fate, many cell divisions become oriented perpendicular to the embryo surface. Daughter cells that are pushed to the inside become ICM and subsequently divide with random orientation. Interestingly, at 16-cell stage already 13% of the blastomeres contribute only to the ICM lineage but only around 5% of the blastomeres would be expected in a fully internal position in the embryo if simple spherical geometrical constrains are assumed (see following paragraph and ref. 4). It is tempting to speculate that this difference is caused by the observed bias towards a perpendicular division axis. Daughter cells that remain outside at the 16-cell stage can then either continue to divide in the plane of the embryo surface and contribute to TE, or divide perpendicularly to the embryo surface with those pushed to the inside adding to the ICM and those remaining outside becoming TE. This model would suggest that the critical step in cell fate specification is the appearance of non-random division angles at the 8- to 16-cell division, which would ensure a higher number of blastomeres is pushed to the inside of the embryo than would be expected solely by the spherical geometry of the embryo, where they are committed to become ICM.
Estimation of the number of cells inside of the embryo if only geometrical constraints were taken into account

To estimate the number of cells that would be located inside of the embryo, i.e. would have no contact to the outside, we used the reasoning and calculations described below.

The mouse embryo at \( n \) cell stages was approximated by \( n \) spheres representing the blastomeres as densely packed as possible inside another sphere representing the zona pellucida. We used numerical solutions of this well-known packing problem calculated by Huang et al.\(^4\) using a global search strategy.

If at \( n \) cell stage the radii of spheres inside a unit sphere are \( r_n \), then the unit sphere of volume \( V_{total} \) can be split into two sub-volumes \( V_{in,n} \) (red) and \( V_{out,n} \) (blue) as illustrated below.

\[
V_{total} = \frac{4}{3} \pi 0.5^3 = 0.523599 \\
V_{in,n} = \frac{4}{3} \pi (0.5 - 2r_n)^3 \\
V_{out,n} = V_{total} - V_{in,n}
\]

The percentages of cells inside and outside at \( n \) cell stage were approximated by the ratios of the total sphere volume to volumes \( V_{in,n} \) and \( V_{out,n} \), and calculated in the table below for selected values of \( n \).

<table>
<thead>
<tr>
<th>( n )</th>
<th>( r_n )</th>
<th>( V_{in,n} )</th>
<th>( V_{out,n} )</th>
<th>( in_n ) [%]</th>
<th>( out_n ) [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.25</td>
<td>0</td>
<td>0.523599</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
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<td>0.02829</td>
<td>0.495308</td>
<td>5.403081</td>
<td>94.59692</td>
</tr>
<tr>
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<td>0.064833</td>
<td>0.458765</td>
<td>12.38226</td>
<td>87.61774</td>
</tr>
</tbody>
</table>
Supplementary Discussion

Comparison to other available light-sheet microscopes

A number of light-sheet microscopes with diverse geometrical configurations have been published and some of them are available commercially. Below we provide a detailed comparison of available microscopes to the light-sheet microscope presented in this work.

The original implementation of light-sheet microscope\(^6\) uses two perpendicular objectives oriented horizontally to illuminate and image a sample that is held vertically at the intersection of the focal planes of these objectives. This design led to the use of agarose or other embedding methods\(^6\) to hold the sample in the narrow space between the objectives. The requirement of sample embedding is one drawback of this design and precludes imaging of many biological samples under physiological conditions. In the field of mouse embryology two studies succeeded to overcome this limitation. Udan and colleagues\(^1\) used hollow agarose cylinders to hold non-embedded mouse post implantation embryos. While this approach overcomes the requirement to embed the sample it is not possible to image many embryos in parallel and use the standard microdrop culture required for mouse pre-implantation embryos. Ichikawa and colleagues\(^2\) replaced the agarose cylinder by an acrylic rod with holes on the side in which embryos were held by their Reichert’s membrane at the ectoplacental cone. This technique is however restricted to samples of a particular geometry and is also not suitable for pre-implantation stages.

Leica Microsystems has recently released a light-sheet add-on module (DLS) to an inverted confocal laser scanning microscope that can achieve light-sheet imaging of non-embedded samples. DLS relies on a mirror attached to a secondary condenser-mounted imaging objective that is dipped into the sample dish from the top. The laser beam from the traditional inverted confocal scanning microscope is reflected from this mirror into the focal plane of the secondary imaging/mirror holding objective. This configuration is technically very different from our light-sheet instrument and has a number of important drawbacks: (1) The detection objective and mirror are dipped into the culture medium and are thus in direct contact with the sample which precludes use of the standard microdrop mammalian embryo culture. (2) Although the sample can be held by gravity only, multi-positioning experiments are difficult because of liquid flows, induced by the secondary objective when the sample dish moves between positions, which can displace non-embedded samples. (3) Dipping the detection objective in the culture medium prevents not only microdrop culture but also any kind of physical separation of the culture medium holding adjacent samples which is essential to achieve different conditions (e.g. drug treatments) in multi-positioning experiments. Thanks to its different design our inverted light-sheet instrument suffers from none of these drawbacks. The limitations described for the Leica DLS module also apply for implementations where imaging and illumination objectives are dipped from top into the dish with culture medium\(^5,6\).

Geometrically similar implementation to the one presented in this study has been recently published by McGorty and colleagues\(^7,8\), enabling imaging of a conventionally mounted samples. This microscope uses a “water prism” to guide the excitation light to and the emitted light from the sample. The tilted coverslip that is unavoidable in this configuration introduces significant astigmatic aberrations and requires the use a cylindrical lens to at least partially compensate for them. These significant aberrations inherent in the optical design are a major limitation of this design and make the use of high NA objectives difficult. In contrast, the design of our sample holder in combination with an immersion water reservoir which holds the water dipping objectives avoids most optical aberrations thanks to refractive index matching and enables the use of high NA objectives.

Range of samples suitable for inverted light-sheet microscope

Compared to the traditional design of light-sheet microscope\(^6\) sample rotation is not possible in our implementation of this microscopy techniques which can be a drawback in particular for larger and strongly scattering samples. This design choice was made to realize important advantages in biological sample handling offered by our setup as discussed in relation to other microscope systems in the previous sections. Our microscope is applicable to a large variety of small to medium sized samples that require specific culture conditions, ranging from single mammalian cells to embryos of various species and organoid models. These samples cover a large range of biological applications and are typically highly transparent, making rotation less essential than preserving physiological culture conditions. In addition, our sample holder design enables quick sample mounting which is critical for many biological experiments such as for example imaging of quickly developing early C. elegans embryos. Although we did not present this data in our study, our microscope is also very well suited to conduct long-term, low light-dose time-lapse imaging of mammalian cells in culture, probably the most widely used model system in biological sciences.

Besides mouse pre-implantation embryos, our microscope is well suited also to image post-implantation embryonic stages, which do not support embedding in agarose gel that would restrict their growth. In contrast to the work by Ichikawa and colleagues\(^2\) as well as by Udan and colleagues\(^1\) many post-implantation embryos can be easily mounted and imaged together thanks to the convenient sample holder. Because our microscope does not support sample rotation (which is however neither possible in the setup of Ichikawa and colleagues) it will be suited mostly for experiments where imaging of larger number of embryos from only one side is needed. In
addition, imaging of embryonic stages that would be difficult to mount into the holes of an acrylic rod will be possible (i.e. in vitro cultured peri-implantation embryos).

Sample holder design
The sample holder has been designed to be microscope slide-sized and quickly mountable on the 3D positioning stage. By refractive index matching of its FEP sample channel it effectively achieves that the sample is optically “flying” in space at a stable position where it’s held by gravity only, in the absence of any mechanical constraint. This sample holder design is required for our setup but with an appropriate adaptor it can in principle be used on any microscope stage that positions a sample above the imaging objective and because of its standard size can easily be adapted to other inverted systems that require refractive index matched sample holding and access from multiple angles.
Supplementary Note

Statistical analysis of lineage segregation

The following statistical test was used to estimate a probability that the observed lineage segregation at a given time occurred by change. Because none of the classical statistical tests could be applied to this problem a permutation test was used. In this test an experimental observation is compared to a large number of randomized cases (simulations). The estimated probability that such observation occurred by chance is given by the ratio of the number of times a value for a randomly generated case was equal or larger than a value for the experimental case to the total number of simulations. To execute this test we first defined a quantitative value representing lineage segregation (variable $\chi^2$ below) at a given time as a sum (for all tree branches at a given time-point) of the normalized squares of the observed number of ICM cells minus the expected number of ICM cells. If we for example observed a tree with 32 ICM and 32 TE cells at the bottom and were to analyze lineage segregation at 4 cell stage then the expected number of ICM cells in each branch would be 8. If in this observed tree we found at 4-cell stage the expected 8 ICM cells in each branch the value for lineage segregation would be zero. If in contrast two of the branches contained all ICM cells segregated together this value would be high. Having this quantitative description of our phenomena of interest, it is possible to compare its value for the observed tree with values obtained for a large number of randomized cases (simulations). To generate a tree with random lineage segregation we have preserved the observed tree topology and the number of TE and ICM cells, but permuted their position at the bottom of the tree. This method effectively estimates numerically the distribution of the quantitative representation of, in our case, lineage segregation and was used because this distribution cannot or would be difficult to derive analytically.

The above described test is formalized below. Cells on the last time point of the lineage tree were assigned ICM or TE fates. For each time-point $t$, the following algorithm was used to perform a permutation test for a null hypothesis that at a time $t$ a lineage segregation is random.

For each time point:

Calculate chi-square statistics for observed lineage tree ($X^2_t$).

$$X^2_t = \sum_{i=1}^{n_b} \frac{((ICM_{t,i}+0.3D_{t,i})-E_{t,i})^2}{E_{t,i}},$$

where $E_{t,i} = \frac{ICM+0.3D}{N} N_{t,i}$ is the expected average number of ICM cells in branch $i$ at time-point $t$.

Permute cells at the bottom of lineage tree (positions of dead cells are kept), calculate chi-square statistics for permutation $P(X^2_{t,p})$ and calculate $I_{t,p} = \begin{cases} 1, & X^2_{t,p} \geq X^2_t \\ 0, & \text{otherwise} \end{cases}$

Repeat previous step $P$ times ($P = 100,000$ in our case).

Final probability that observed lineage segregation at time point $t$ is random is:

$$p_t = \frac{\sum_{i=1}^{P} I_{t,i}+1}{P+1}$$

This calculation was executed for each time-point and results for one time-point from each level of the tree are shown in Supplementary Fig. 8. Above symbols denote to following quantities:

- $N$: Total number of cells.
- $B_t$: Number of branches at time-point $t$.
- $n_{t,i}$: Number of cells in branch $i$ at time-point $t$.
- $ICM$: Total number of ICM cells.
- $icm_{t,i}$: Number of ICM cells in branch $i$ at time-point $t$.
- $D$: Total number of dead cells.
- $D_{t,i}$: Number of dead cells in branch $i$ at time-point $t$.

Note that the number of dead cells $D$, which have no assigned fate, is in the equation for $E_{t,i}$ multiplied by a factor of 0.3 which roughly represent the average relative number of ICM cells. The exact relative number of ICM cells in our dataset is 0.36. We have therefore performed the above test using also a factor of 0.4 which lead to an identical conclusion showing that the above described test is not sensitive to this factor. Test was implemented in Python using numpy and scipy libraries.

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Calculation of combined probability by Fisher’s combined probability test

The above statistical analysis was executed for each lineage tree (embryo) independently. To detect even a slight bias in lineage segregation at a given cell stage, results of all embryos were combined using Fisher’s combined probability test. Probabilities of time-points with 2, 4, 8, 16 and 32 cells were used.

\[ p_{i,n} \] Probability obtained by the above described test for embryo \( i \) at \( n \)-cell stage.

\[ n \in \{4, 8, 16, 32\} \] Number of cells (branches) in embryo at \( n \)-cell stage.

\[ K \] Total number of embryos.

\[ \chi^2_n = -2 \sum_{i=1}^{K} \ln(p_{i,n}) \] Chi-square statistics for all embryos at \( n \)-cell stage.

\[ p_n = 1 - \text{chi2.cdf}(\chi^2_n, 2K) \] Combined probability for all embryos at \( n \)-cell stage.

To calculate chi-square cumulative distribution function we used a stats.chi2.cdf method from the Python scipy package.
References